MycoKeys 9: 11–28 (2015)
doi: 10.3897/mycokeys.9.9032
http://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Buwchfawromyces eastonii gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces

Tony Martin Callaghan[1], Sabine Marie Podmirseg[2], Daniel Hohlweck[3], Joan Elizabeth Edwards[1], Anil Kumar Puniya[4], Sumit Singh Dagar[5], Gareth Wyn Griffith[1]

1 *Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Adeilad Cledwyn, Penglais, Aberystwyth, Ceredigion SY23 3DD, Wales, UK* **2** *Universität Innsbruck, Institut für Mikrobiologie, Technikerstraße 25d, A-6020 Innsbruck, Austria* **3** *Department of Applied and Molecular Microbiology, Technische Universität Berlin, Straße des 17. Juni 135 D-10623, Berlin, Germany* **4** *ICAR-National Dairy Research Institute, Karnal, India* **5** *Agharkar Research Institute, Pune, Maharashtra, India*

Corresponding author: *Gareth Wyn Griffith* (gwg@aber.ac.uk)

Academic editor: *T. Lumbsch* | Received 27 November 2014 | Accepted 10 February 2015 | Published 4 March 2015

Citation: Callaghan TM, Podmirseg SM, Hohlweck D, Edwards JE, Puniya AK, Dagar SS, Griffith GW (2015) *Buwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces. MycoKeys 9: 11–28. doi: 10.3897/mycokeys.9.9032

Abstract

The novel anaerobic fungus *Buwchfawromyces eastonii* **gen. nov.**, **sp. nov.**, belonging to order Neocallimastigales (phylum Neocallimastigomycota) is described. Morphologically similar to *Piromyces* but genetically quite distinct, this fungus (isolate GE09) was first isolated from buffalo faeces in west Wales and then subsequently isolated from sheep, cattle and horse in the same area. Phylogenetic analysis of LSU and ITS sequence confirmed that *B. eastonii* isolates formed a distinct clade close to the polycentric *Anaeromyces* spp. The morphology of GE09 is monocentric with monoflagellate zoospores. However, the sporangial stalk (sporangiophore) is often distinctly swollen and the proximal regions of the rhizoidal system twisted in appearance.

Key words

Ruminant, symbiosis, fungal phylogenetics, buffalo, DNA barcoding

Copyright *Tony Martin Callaghan et al.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Introduction

At present six genera of anaerobic fungi are recognised, differentiated by thallus morphology and zoospore flagellation (Gruninger et al. 2014). However, the lack of other reliable morphological features, combined with difficulties in the cultivation of these fungi, exchange of cultures between different labs and their morphological variability in culture, has hindered more definitive study of their diversity. As the amount of DNA barcode data (mostly based on the internal transcribed spacer regions, ITS1 and ITS2), both for pure cultures and from environmental DNA sequencing projects has increased, it has become apparent that the six existing clades represent only a subset of the full diversity of the anaerobic fungi (Griffith et al. 2010). For instance, Liggenstoffer et al. (2010) generated 250,000 ITS1 sequences from a diverse range of host species and identified eight potentially novel clades. Furthermore, reconciliation of morphological features and DNA barcode data has led to the reassignment of some taxa (Fliegerová et al. 2012). Following revision of the taxonomy of kingdom Fungi, the anaerobic fungi are now assinged to phylum Neocallimastigomycota (Hibbett et al. 2007). However, the status of the anaerobic fungi as a distinct phylum as opposed to a class within phylum Chytridiomycota remains a matter of debate (Frey 2012; Powell and Letcher 2014).

The genus *Anaeromyces* was first discovered by Breton et al. (1990), describing *Anaeromyces mucronatus*. A near-simultaneous publication by Ho et al. (1990) named a morphologically similar fungus as *Ruminomyces elegans* but in recognition of the rules of priority, Ho and Barr (1993a) later renamed this species as *A. elegans*. Neither viable cultures nor DNA from these original isolates remains (Prof. Yin Wan Ho and Dr. Brigitte Gaillard-Martinie, pers. comms.), though the >80 ITS sequences submitted to GenBank under this genus name suggests that cultures conforming to the morphological description of *Anaeromyces* (uniflagellate zoospores, polycentric mycelium) are commonly isolated from the digestive tracts of different types of herbivorous mammals.

The taxonomic status of one isolate (GE09) is addressed, for which data were previously submitted to GenBank (five ITS cloned from this single isolate; EU414755–EU414759) under the generic name *Anaeromyces* (Edwards et al. 2008). More detailed genetic analysis has been conducted to clarify its taxonomic position, along with its morphological characterisation. Microscopic examination and phylogenetic reconstruction, using both the ITS1 and LSU regions of the rRNA locus, showed that GE09 and three other similar isolates (isolated from different host species) were clearly distinct from the main *Anaeromyces* clade. We therefore conclude that these four isolates represent a new genus, which we name *Buwchfawromyces* gen. nov.

Methods

Enrichment culture from faeces

Anaerobic fungal cultures were enriched using a basal medium (Davies et al. 1993; Orpin 1977) supplemented with milled wheat straw. The basal medium was made up as follows: 150 ml salts solution 1 (3 $g.L^{-1}$ K_2HPO_4 in distilled water), 150 ml salts solution 2 (3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4.7H_2O$ and 0.5 g $CaCl_2$; dissolved in 1 L distilled water, in that order), yeast extract (3 g; Oxoid, Basingstoke, UK), tryptone (10 g; Fisher Scientific Ltd., Loughborough, UK), resazurin (2 ml of 0.1% solution) and hemin (2 ml of 0.05% solution dissolved in 1:1 ethanol / 50 mM NaOH) were added and the volume made up to 850 ml with distilled water. After boiling (until light red in colour) and cooling, 150 ml centrifuged (clarified) rumen fluid, 6 g $NaHCO_3$ and 1 g L-cysteine-HCl were added (final volume 1 L). The resultant solution was then deoxygenated by gassing with CO_2 for 1 h. The medium was dispensed anaerobically in 9 ml aliquots into 15 ml Hungate tubes containing milled wheat straw (5 $mg.ml^{-1}$; 0.5% w/v; dry-sieved through 2 mm mesh). Tubes were then capped with a polypropylene bung held in place by a screw cap, and autoclaved (121 °C/15 min).

Freshly voided faecal samples (ca. 20 g) were collected and transported to the laboratory within 1 h. Aliquots (ca.10 g fresh matter) of these samples were then homogenised for 2 min with 90 ml of pre-warmed (39 °C) basal medium (without wheat straw, yeast extract or tryptone) in a pre-sterilised Stomacher 400 Circulator Bag (polythene; 177 × 305 mm) using a Seward Stomacher 400 Circulator Paddle Blender (Seward Ltd., Worthing, W. Sussex, UK). A 10-fold serial dilution of this faecal homogenate was then prepared in pre-warmed basal medium (1 ml transferred to 9 ml basal medium in 15 ml Hungate tube). The 10^{-4}, 10^{-5} and 10^{-6} dilutions were used to inoculate (1 ml) the tubes of basal medium (9 ml) supplemented with wheat straw. A mixed solution of penicillin, ampicillin and streptomycin sulphate in 50% (v/v) ethanol (5 $mg.ml^{-1}$ of each; 10 $ml.L^{-1}$ added to give a final medium concentration of 50 $\mu g.ml^{-1}$) was also added to tubes before they were recapped, in order to inhibit bacterial growth. Tubes were incubated in the dark at (39 °C), and routine subculture was conducted at 3–5 d intervals. Exposure of the samples to oxygen was prevented by undertaking the manipulations in a box flushed with CO_2. Cultures were also grown on basal medium containing cellobiose (5 $mg.ml^{-1}$) instead of wheat straw. Purity of the isolates was ensured by three cycles of cultivation in roll tubes (Joblin 1981), with well-separated colonies being excised for subculture with a mycological spear.

For cryopreservation of cultures, a method based on the procedures suggested by Sakurada et al. (1995) and Yarlett et al. (1986) was devised. A 5× cryopreservation solution was prepared by mixing 49.7 g ethylene glycol with 155 ml clarified rumen, 200 µl of 0.1% resazurin, 0.2 g L-cysteine and 1.2 g $NaHCO_3$ under anaerobic conditions (total volume 200 ml; 3.2 M ethylene glycol). This solution was bubbled with CO_2 for ca. 3 h, prior to anaerobically dispensing 10 ml aliquots into Hungate tubes which were autoclaved and stored at -20 °C until use. The 5× cryopreservation solution was added

to 3–5 d old wheat straw cultures (2.5 ml per 10 ml culture) under anaerobic conditions in Hungate tubes. After mixing, tubes were chilled in ice water for 15 min and then anaerobically dispensed in 2 ml aliquots into sterile 2 ml cryovials. Cryovials were placed at -80 °C overnight, before being transferred to liquid nitrogen for longer-term storage. Storage of cryopreserved cultures at -80 °C is possible for up to a few months but for prolonged storage, liquid nitrogen was found to be much more reliable.

Microscopy

Microscopy was conducted on cultures, grown on either wheat straw or cellobiose, using an epifluorescence microscope (Olympus BX50) with images recorded using a Nikon Coolpix 995 digital camera. For visualisation of nuclei, DAPI (0.3 $mg.ml^{-1}$ in 50 mM Tris-HCl, pH 7.2) was added, and for enhanced definition of cell walls and septa, Calcofluor white M2R (100 μM; Day et al. 2002) was used (UV-W filters: 365 nm excitation/420 nm emission).

Phylogenetic analysis

DNA extraction was carried out using the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987), with modifications as described by Griffith and Shaw (1998). Cultures were harvested after 3–6 d incubation, and the biomass washed three times with sterile distilled water before being freeze–dried and ground to a powder. Ground biomass (ca. 50 mg) was used for DNA extraction, and the purified DNA resuspended in 50 μl TE buffer (pH 8.0) before being stored at -20 °C.

For genetic analysis, the D1/D2 domain of large-subunit (LSU) ribosomal DNA and internal transcribed spacer 1 (spanning ITS1 and ITS2) were amplified, using the primer pairs NL1 (GCATATCAATAAGCGGAGGAAAAG) / NL4 (GGTCCGTGTTTCAAGACGG) (Dagar et al. 2011; Fliegerová et al. 2006) and GM1 (TGTACACACCGCCCGTC) / MN106 (CGTTGTAAAACACTCAWAACC) (Edwards et al. 2008), respectively. Sequence management was conducted within the Geneious (v6.1.6) bioinformatics package (Drummond et al. 2011), using MAFFT (Katoh et al. 2002) for sequence alignment (default settings).

Phylogenetic reconstruction was conducted using TOPALi (v2.5) (Milne et al. 2004). For LSU analysis, maximum likelihood analysis was conducted using PhyML (Guindon et al. 2010) and the TrN+gamma substitution model recommended by TOPALi. For analysis of the ITS, only the ITS1 region was used since the great majority of available sequences cover only this region (and not ITS2). To establish the phylogenetic position of isolate GE09, ITS1 sequences derived from five clones were aligned with environmental and isolate sequences belonging to the four closely related monoflagellate genera. The resulting MAFFT alignment was trimmed to include only 15 bp of the flanking 18S and 5.8S regions, and duplicate sequences were removed

(retaining sequences relating to cultured fungi, if present). For ITS1 analysis, maximum likelihood analysis was conducted using PhyML (Guindon et al. 2010) and the GTR substitution model recommended by TOPALi. Both the ITS1 and LSU alignments have been submitted to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S16672).

Results and discussion

Ecology

The isolate GE09 was originally isolated from the faeces of a domesticated Asian water buffalo (*Bubalus bubalis*) on 26th Feb 2004 at Panthwylog Farm, Llanon, Ceredigion, Wales (N52.279; W-4.169). The buffalo was part of a herd kept outdoors, maintained on grass pasture supplemented with grass silage. More recently, three additional pure cultures (each from a different host species have been isolated), all identical in morphology and LSU DNA barcode to GE09. These isolates were obtained as follows: from cow (*Bos taurus*) faeces (isolate HoCal4.C3.3; Nant yr Arian, Ponterwyd, Ceredigion; 6th Feb 2013; N52.416; W-3.891), sheep (*Ovis aries*) faeces (HoCal4.B3c, also Nant yr Arian, 6th Feb 2013) and horse (*Equus ferus caballus*) faeces (isolate HoCal4.D1.2; Aberystwyth University Lluest livery yard; 6th Feb 2013; N52.410; W-4.051).

Morphology

Thalli of isolate GE09, when grown on wheat straw or cellobiose as a carbon source, were consistently monocentric, with rhizoids radiating from a single developing sporangium. Mature sporangia were spherical to ovoid 30 to 80 μm long and 20 to 60 μm wide (Fig. 1). No apical projections, as found in *Anaeromyces mucronatus* (Breton et al. 1990) or *Piromyces mae* (Li et al. 1990) (referred to as a mucro or papilla respectively by these authors), were observed.

Zoospores (spherical; diameter 9–11 μm) were readily observed in 3–5 d old cultures grown on wheat straw and consistently bore a single flagellum (30–40 μm long; 3–4× longer than the length of the zoospore body). However, the process whereby zoospores were released from the sporangium was not observed. On a single occasion, a large (30 μm diameter) zoospore-like structure bearing numerous flagella, each emerging from a different point on the zoospore body, was detected (Suppl. material 1A). This is possibly the result of the agglomeration and fusion of numerous zoospores (hence the numerous flagella that are visible), similar to the structure previously observed by Orpin (1975) in *N. frontalis* (Orpin's Fig. 2; Suppl. material 1B).

The most distinctive feature of the thalli of GE09 were the swollen sporangiophores (40–80 μm long and 15–50 μm wide), occasionally comparable in volume to the sporangium they supported (Fig. 1E–H). Also visible was a septum at the point

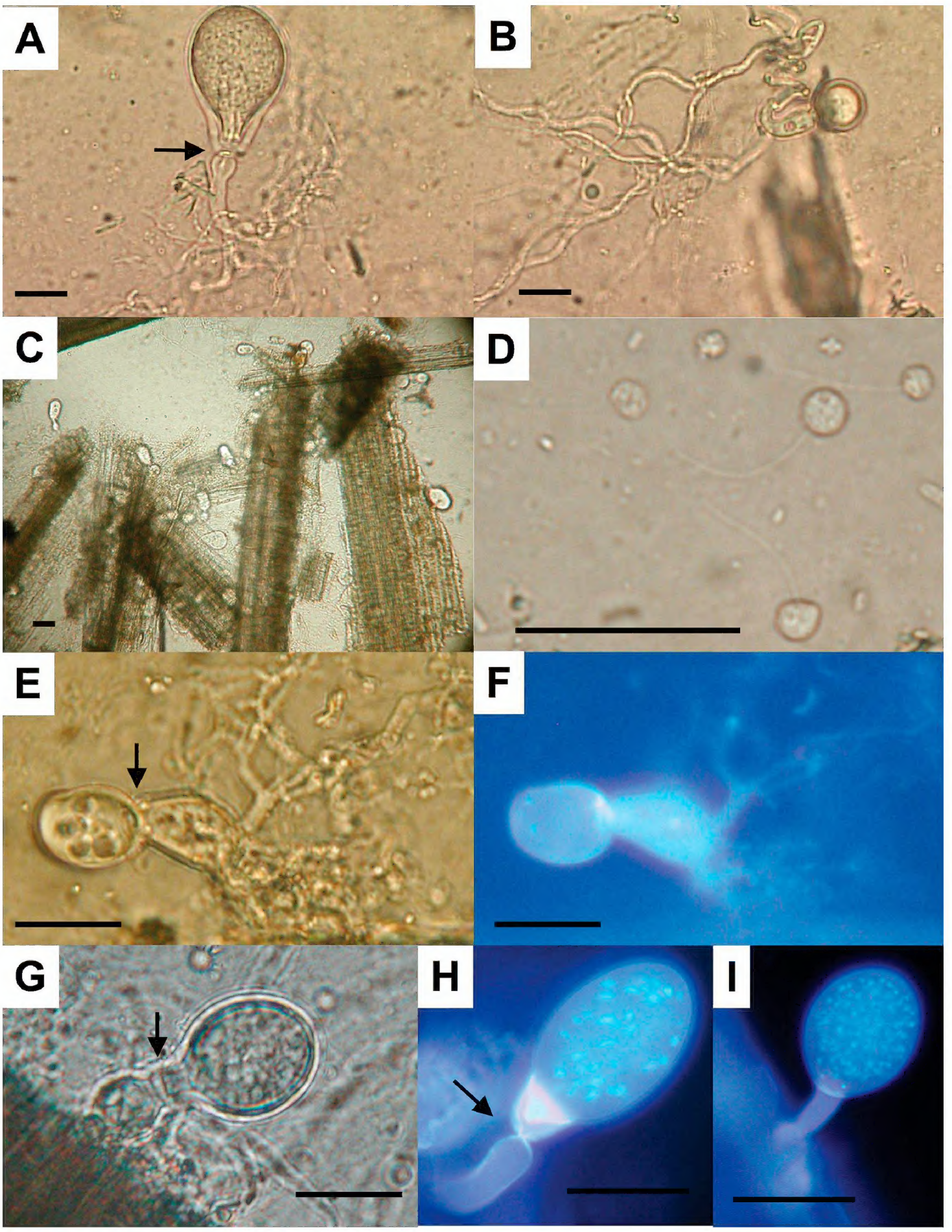

Figure 1. Morphology of *Buwchfawromyces eastonii*. Sporangia are ovoid (**A**) to spherical (**B**), tending to be more elongate when growing on straw particles (**C**). Zoospores are uniformly monoflagellate (**D**). A distinct septum is visible where the sporangium is attached to the sporangiophore (**A, E, G, H** arrowed) and sporangiophores are often swollen (**E–H**). Nuclei were not observed in sporangiophores or rhizoids (**F, H, I**). Scalebar indicates 50 μm.

where the sporangium joined the sporangiophore (Fig. 1A, E–I). The sporangiophore was contiguous with the rhizoids which tapered (from 20 μm to 5 μm) and branched (Fig. 1B). The proximal rhizoids (within 100 μm of the sporangium) were often contorted (Fig. 1B). DAPI staining was used to observe the location of nuclei within the thalli (Fig. 1F, H, I); these were abundant in sporangia but none were observed in the sporangiophores or rhizoids.

The swollen sporangiophores and twisted rhizoids observed here are very similar to those noted by Ho et al. (1993b) in *Piromyces spiralis* (Suppl. material 1C, D). Also similar are the swollen sporangiophores reported in *P. mae* (Li et al. 1990) (isolate PN11 from horse; Fig. 25). It is also noteworthy that *Anaeromyces* (formerly *Piromyces*) *polycephalus* (Suppl. material 1E) (Chen et al. 2002; Kirk 2012), whilst forming multiple rather than single sporangia, also forms a distinctly swollen sporangiophore. It is possible that such structures play some role in physical disruption of the substrate, as is the case for the bulbous holdfasts formed by *Caecomyces* and *Cyllamyces* spp.

Cultures of GE09 maintained viability, and could be subcultured, after incubation at 39 °C for periods of several weeks. This raised the possibility that these cultures may form long-term survival structures (McGranaghan et al. 1999; Struchtemeyer et al. 2014). Thick-walled and septate structures (3–4 septa; 30–40 μm long × 10–15 μm wide), very similar to those previously observed in *Anaeromyces* sp. EO2 (Brookman et al. 2000) were seen in wheat straw cultures incubated for 28 d (Suppl. material 1F, G) and never observed in younger (3–5 d old) cultures. However, detailed examination of the development of these putative resting structures was not undertaken.

Phylogenetic analysis

Detailed examination of isolate GE09 was not undertaken when it was first isolated. However, it was used as a reference sample in a study of the colonisation of forage by anaerobic fungi (Edwards et al. 2008), in the course of which the ITS1/2 spacer regions were amplified and cloned. The sequences of the five clones were submitted to GenBank (EU414755–EU414759) under the generic name *Anaeromyces*, since these sequences clustered close to the *Anaeromyces* clade at that time. However, more detailed analysis has since suggested that this isolate is quite distinct from *Anaeromyces* (Kittelmann et al. 2012).

The sequence of the D1/D2 domains (ca. 750 bp) of the LSU of GE09 and the three other isolates were identical (submitted to GenBank as KP205570). These sequences were aligned with 36 other LSU sequences (from GenBank) covering all the known genera of Neocallimastigomycota (700 bp alignment; 188 phylogenetically informative sites), and including the outgroup taxon *Gromochytrium mamkaevae* (Chytridiomycota). Phylogenetic reconstruction consistently recovered *Buwchfawromyces* isolates as a distinct clade (85% bootstrap support). LSU sequences from *Anaeromyces*, *Neocallimastix* and *Orpinomyces* were also recovered as distinct clades with strong (≥80%) bootstrap support (Fig. 2). *Neocallimastix* and *Orpinomyces* were more

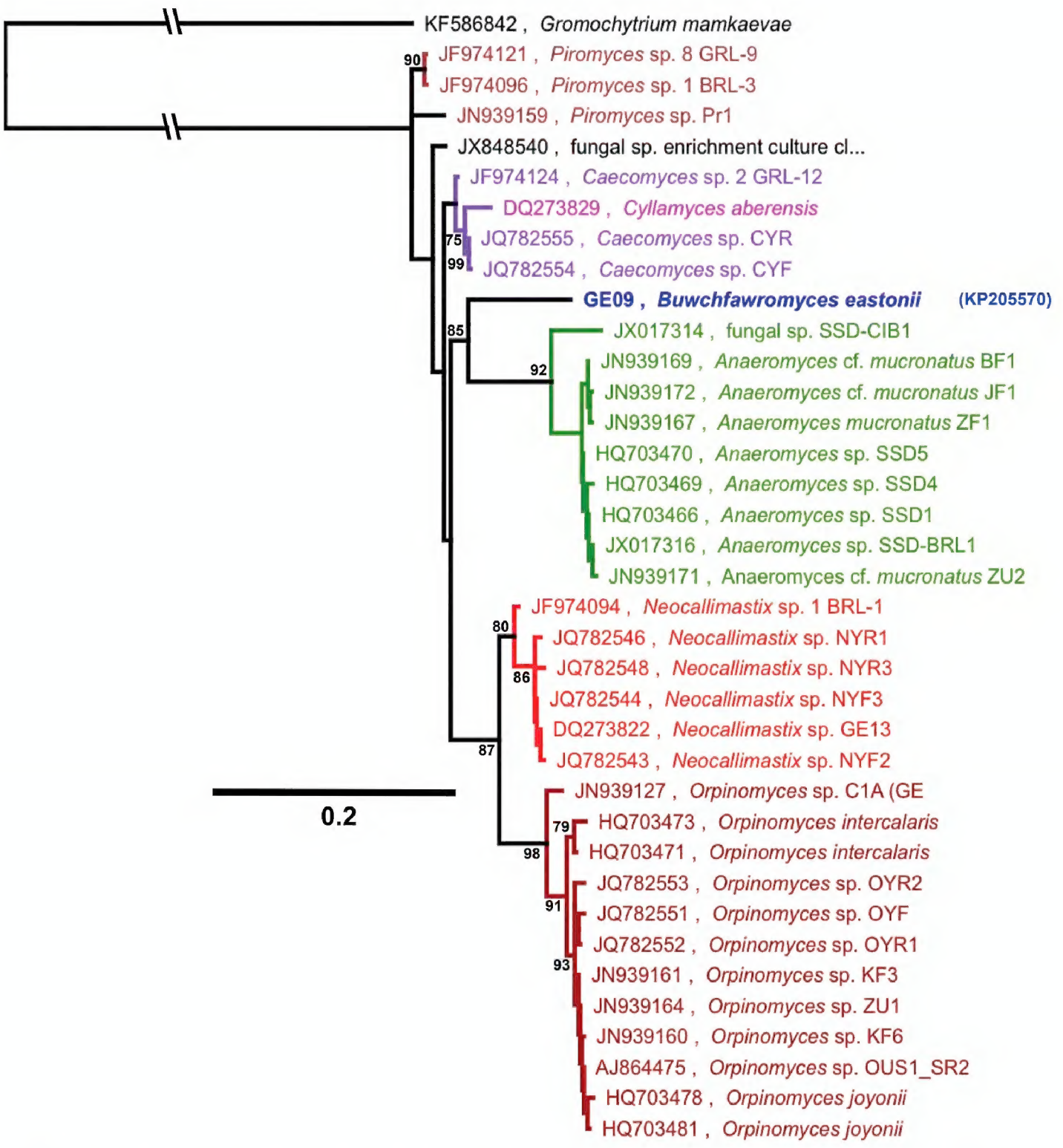
KF586842 , Gromochytrium mamkaevae
90
JF974121 , Piromyces sp. 8 GRL-9
JF974096 , Piromyces sp. 1 BRL-3
JN939159 , Piromyces sp. Pr1
JX848540 , fungal sp. enrichment culture cl...
JF974124 , Caecomyces sp. 2 GRL-12
DQ273829 , Cyllamyces aberensis
75
JQ782555 , Caecomyces sp. CYR
99
JQ782554 , Caecomyces sp. CYF
GE09 , Buwchfawromyces eastonii (KP205570)
85
JX017314 , fungal sp. SSD-CIB1
92
JN939169 , Anaeromyces cf. mucronatus BF1
JN939172 , Anaeromyces cf. mucronatus JF1
JN939167 , Anaeromyces mucronatus ZF1
HQ703470 , Anaeromyces sp. SSD5
HQ703469 , Anaeromyces sp. SSD4
HQ703466 , Anaeromyces sp. SSD1
JX017316 , Anaeromyces sp. SSD-BRL1
JN939171 , Anaeromyces cf. mucronatus ZU2
JF974094 , Neocallimastix sp. 1 BRL-1
80
JQ782546 , Neocallimastix sp. NYR1
86
JQ782548 , Neocallimastix sp. NYR3
JQ782544 , Neocallimastix sp. NYF3
DQ273822 , Neocallimastix sp. GE13
87
JQ782543 , Neocallimastix sp. NYF2
JN939127 , Orpinomyces sp. C1A (GE
79
HQ703473 , Orpinomyces intercalaris
98
HQ703471 , Orpinomyces intercalaris
JQ782553 , Orpinomyces sp. OYR2
91
JQ782551 , Orpinomyces sp. OYF
JQ782552 , Orpinomyces sp. OYR1
93
JN939161 , Orpinomyces sp. KF3
JN939164 , Orpinomyces sp. ZU1
JN939160 , Orpinomyces sp. KF6
AJ864475 , Orpinomyces sp. OUS1_SR2
HQ703478 , Orpinomyces joyonii
HQ703481 , Orpinomyces joyonii
0.2

Figure 2. Maximum likelihood tree based on alignment of the D1/D2 region of the Large Ribosomal Subunit (700 bp alignment; 37 sequences; 188 phylogenetically informative sites; TrN+gamma model). Bootstrap values over 70% are shown (1000 replicates). Scale bar indicates number of substitutions per site.

closely related to each other than to other genera, consistent with the occurrence of polyflagellate flagella in these genera, a feature not found in other flagellate fungi (James et al. 2006). The genera forming bulbous holdfasts (*Caecomyces*, *Cyllamyces*) also formed a distinct clade, and *Piromyces* isolates occupied a basal position but without strong bootstrap support.

Whilst analysis of the LSU proved informative to confirm the distinctiveness of the GE09 clade, there are relatively few LSU sequences available in GenBank for comparison. Therefore, phylogenetic analysis of the ITS1 internal transcribed spacer region, for which there are hundreds of published sequences, was conducted (357

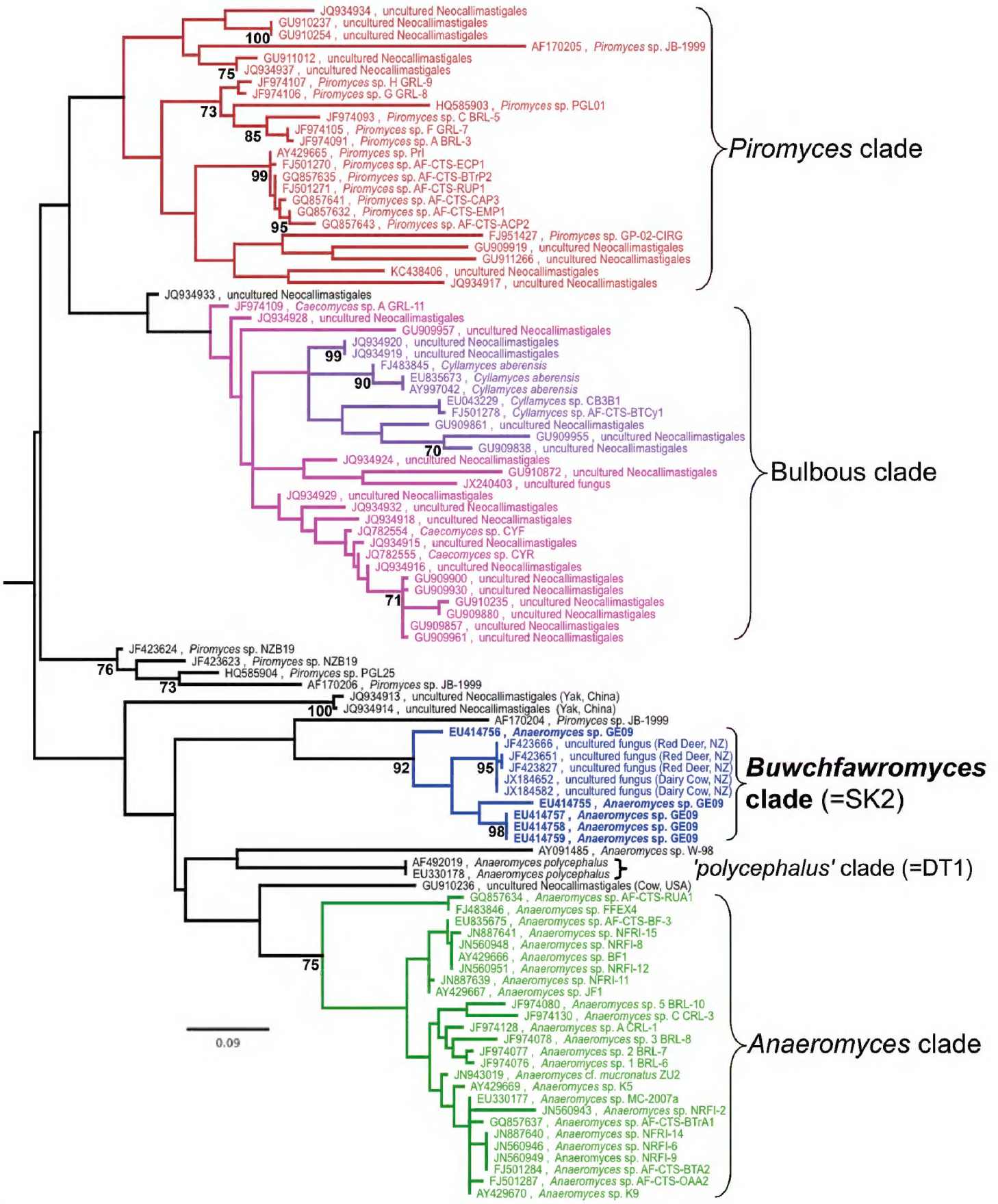
JQ934934 , uncultured Neocallimastigales
GU910237 , uncultured Neocallimastigales
100
GU910254 , uncultured Neocallimastigales
AF170205 , Piromyces sp. JB-1999
GU911012 , uncultured Neocallimastigales
75
JQ934937 , uncultured Neocallimastigales
JF974107 , Piromyces sp. H GRL-9
JF974106 , Piromyces sp. G GRL-8
HQ585903 , Piromyces sp. PGL01
73
JF974093 , Piromyces sp. C BRL-5
85
JF974105 , Piromyces sp. F GRL-7
JF974091 , Piromyces sp. A BRL-3
AY429665 , Piromyces sp. PrI
FJ501270 , Piromyces sp. AF-CTS-ECP1
99
GQ857635 , Piromyces sp. AF-CTS-BTrP2
FJ501271 , Piromyces sp. AF-CTS-RUP1
GQ857641 , Piromyces sp. AF-CTS-CAP3
GQ857632 , Piromyces sp. AF-CTS-EMP1
95
GQ857643 , Piromyces sp. AF-CTS-ACP2
FJ951427 , Piromyces sp. GP-02-CIRG
GU909919 , uncultured Neocallimastigales
GU911266 , uncultured Neocallimastigales
KC438406 , uncultured Neocallimastigales
JQ934917 , uncultured Neocallimastigales
Piromyces clade
JQ934933 , uncultured Neocallimastigales
JF974109 , Caecomyces sp. A GRL-11
JQ934928 , uncultured Neocallimastigales
GU909957 , uncultured Neocallimastigales
JQ934920 , uncultured Neocallimastigales
99
JQ934919 , uncultured Neocallimastigales
FJ483845 , Cyllamyces aberensis
90
EU835673 , Cyllamyces aberensis
AY997042 , Cyllamyces aberensis
EU043229 , Cyllamyces sp. CB3B1
FJ501278 , Cyllamyces sp. AF-CTS-BTCy1
GU909861 , uncultured Neocallimastigales
GU909955 , uncultured Neocallimastigales
70
GU909838 , uncultured Neocallimastigales
JQ934924 , uncultured Neocallimastigales
GU910872 , uncultured Neocallimastigales
JX240403 , uncultured fungus
JQ934929 , uncultured Neocallimastigales
JQ934932 , uncultured Neocallimastigales
JQ934918 , uncultured Neocallimastigales
JQ782554 , Caecomyces sp. CYF
JQ934915 , uncultured Neocallimastigales
JQ782555 , Caecomyces sp. CYR
JQ934916 , uncultured Neocallimastigales
GU909900 , uncultured Neocallimastigales
GU909930 , uncultured Neocallimastigales
71
GU910235 , uncultured Neocallimastigales
GU909880 , uncultured Neocallimastigales
GU909857 , uncultured Neocallimastigales
GU909961 , uncultured Neocallimastigales
Bulbous clade
JF423624 , Piromyces sp. NZB19
JF423623 , Piromyces sp. NZB19
76
HQ585904 , Piromyces sp. PGL25
73
AF170206 , Piromyces sp. JB-1999
JQ934913 , uncultured Neocallimastigales (Yak, China)
100
JQ934914 , uncultured Neocallimastigales (Yak, China)
AF170204 , Piromyces sp. JB-1999
EU414756 , Anaeromyces sp. GE09
JF423666 , uncultured fungus (Red Deer, NZ)
JF423651 , uncultured fungus (Red Deer, NZ)
92
95
JF423827 , uncultured fungus (Red Deer, NZ)
JX184652 , uncultured fungus (Dairy Cow, NZ)
JX184582 , uncultured fungus (Dairy Cow, NZ)
EU414755 , Anaeromyces sp. GE09
EU414757 , Anaeromyces sp. GE09
98
EU414758 , Anaeromyces sp. GE09
EU414759 , Anaeromyces sp. GE09
Buwchfawromyces clade (=SK2)
AY091485 , Anaeromyces sp. W-98
AF492019 , Anaeromyces polycephalus
EU330178 , Anaeromyces polycephalus
'polycephalus' clade (=DT1)
GU910236 , uncultured Neocallimastigales (Cow, USA)
GQ857634 , Anaeromyces sp. AF-CTS-RUA1
FJ483846 , Anaeromyces sp. FFEX4
EU835675 , Anaeromyces sp. AF-CTS-BF-3
JN887641 , Anaeromyces sp. NFRI-15
JN560948 , Anaeromyces sp. NRFI-8
AY429666 , Anaeromyces sp. BF1
75
JN560951 , Anaeromyces sp. NRFI-12
JN887639 , Anaeromyces sp. NFRI-11
AY429667 , Anaeromyces sp. JF1
JF974080 , Anaeromyces sp. 5 BRL-10
JF974130 , Anaeromyces sp. C CRL-3
JF974128 , Anaeromyces sp. A CRL-1
JF974078 , Anaeromyces sp. 3 BRL-8
JF974077 , Anaeromyces sp. 2 BRL-7
JF974076 , Anaeromyces sp. 1 BRL-6
JN943019 , Anaeromyces cf. mucronatus ZU2
AY429669 , Anaeromyces sp. K5
EU330177 , Anaeromyces sp. MC-2007a
JN560943 , Anaeromyces sp. NRFI-2
GQ857637 , Anaeromyces sp. AF-CTS-BTrA1
JN887640 , Anaeromyces sp. NFRI-14
JN560946 , Anaeromyces sp. NRFI-6
JN560949 , Anaeromyces sp. NRFI-9
FJ501284 , Anaeromyces sp. AF-CTS-BTA2
FJ501287 , Anaeromyces sp. AF-CTS-OAA2
AY429670 , Anaeromyces sp. K9
Anaeromyces clade
0.09

Figure 3. Maximum likelihood tree based on alignment (357 bp) of the ITS1 region. Midpoint rooting was used to root the tree and bootstrap values over 70% are shown (1000 replicates). Scalebar shows the number of substitutions per site. Clades corresponding to the known genera, the new *Buwchfawromyces* clade and also the '*polycephalus*' clade are labelled. Codes in brackets indicate the novel clades identified by Koetschan et al. (2014).

bp alignment; 271 phylogenetically informative sites, 101 sequences) (Fig. 3). Since analysis of the LSU sequences had shown members of the genera *Neocallimastix* and *Orpinomyces* to be quite distinct, these were omitted from analyses of the ITS1 region

in order to improve the quality of the alignments (fewer gaps). Additionally, 'environmental' sequences obtained from clone library studies, (mostly labelled as "uncultured Neocallimastigales") were also included, as recommended by Nilsson et al. (2011).

The *Buwchfawromyces* (GE09) clade was again recovered with high (92%) bootstrap support, as was the *Anaeromyces* clade. Five environmental sequences, all from New Zealand (from cow or red deer; Fig. 2 and listed in Suppl. material 4) also fall into the *Buwchfawromyces* clade. This clade was also identified (and denoted as clade SK2) in the recent study by Koetschan et al. (2014), who were able to create a reliable phylogeny by using predictors of ITS1 folding to decrease the effects of gaps in anaerobic fungal ITS1 alignments.

The largest survey of anaerobic fungi conducted to date is that of Liggenstoffer et al. (2010). They obtained ca. 250,000 ITS1 sequences, from the faeces of 30 herbivore species kept in Oklahoma zoos (Liggenstoffer et al. 2010), including three of the four herbivore species from which we cultured *Buwchfawromyces*. It was surprising that such a large dataset should yield no sequences falling into the *Buwchfawromyces* clade. However, examination of the primers used by Liggenstoffer et al. (2010) revealed the presence of mismatches between the forward primer (MN100modified; 5'-TCCTACCCTTTGTGAATTTG-3') and the cognate sequences found in members of the *Buwchfawromyces* clade (TCCTACCCTTTGTGAATT***GT*** or TCCTTACCCTTTGTGAA***CT******GA***) (Suppl. material 2). These mismatches would very likely have caused significant primer bias and thus poor amplification of any *Buwchfawromyces* spp. present.

Five cloned ITS1/2 PCR amplicons of GE09 were originally submitted to GenBank (EU414755–EU414759). These reveal an extremely high level of sequence divergence (<27 polymorphisms within the ca. 200bp ITS1 region; 87.1%–99.5% identity, (Suppl. material 3). High levels of intragenomic variation has also been found in other anaerobic fungi (Ozkose 2001), and also in some other fungal taxa, for instance phylum Glomeromycota (Pawlowska and Taylor 2004; Pringle et al. 2000). For GE09, the most divergent of these clones (EU414756) was more distantly related to the other GE09 clones than were sequences from New Zealand (Fig. 3). A cut-off level of 97% identity is often used to define species or OTUs (operational taxonomic units) in mycology (Nilsson et al. 2008; Yamamoto and Bibby 2014). However, such high levels of intragenomic variation, make it very difficult to generate reliable species hypotheses (Koljalg et al. 2013) for the Neocallimastigomycota based on ITS sequences alone, although the delineation of different genera is still possible.

We consider that isolate GE09 and the three other similar fungi also isolated in the Aberystwyth area represent a new genus *Buwchfawromyces* within the phylum Neocallimastigomycota. It is not possible for *Buwchfawromyces* to be placed within the related genus *Anaeromyces*, since it forms a monocentric thallus not consistent with the circumscription of this genus (Breton et al. 1990) which comprises species with polycentric thalli. Close to *Buwchfawromyces* and *Anaeromyces*, is the species currently known as *Anaeromyces* (formerly *Piromyces*) *polycephalus*. This species has a distinctive morphology (Suppl. material 1E), with multiple sporangia arising from a swollen spo-

rangiophore, and with an anucleate rhizoidal system. It was originally isolated and described from buffalo in Taiwan (isolate W-33; (Chen et al. 2002)) and has since been reported from India (isolate CTS-47 from zebra faeces; GenBank EU330178); this clade was denoted DT1 (Fig. 3) by Koetschan et al. (2014). Given that '*A. polycephalus*' is both morphologically and genetically distinct, this species should be renamed.

The unusually high level of intragenomic variation in ITS1 makes it difficult to nominate a single reference sequence, therefore two are presented (EU414755 and EU414756).

Diagnosis

***Buwchfawromyces* Callaghan, Tony & G.W. Griff., gen. nov.**
Registration identifier: IF550797

Note. Strictly anaerobic fungus with determinate, monocentric thallus with single, spherical to ovoid terminal sporangium (often with swollen sporangiophore) and forming uniflagellate zoospores. The clade is defined by the sequences EU414755 and EU414756 (ITS1, 5.8S, ITS2 complete), and also KP205570 (LSU, partial sequence). The most genetically similar genus is *Anaeromyces*, which is defined as forming a polycentric thallus (Breton et al. 1990. FEMS Microbiol. Lett. 58, p.177), in contrast to the monocentric *Buwchfawromyces*.

***Buwchfawromyces eastonii* Callaghan, Tony & G.W. Griff., gen. nov., sp. nov.**
Registration identifier: IF550798

Note. An obligately anaerobic fungus with determinate monocentric thallus and spherical to ovoid sporangia. Thalli often with a distinctly swollen sporangiophore and twisted rhizoids. Extensive rhizoidal system but sporangiphore and rhizoids lacking nuclei. Sporangia ovoid to spherical (30–80 μm × 20–60 μm), non-papillate. Zoospores formed abundantly, spherical (9–11 μm diameter) with single flagellum (30–40 μm long). The reference sequences for this species are EU414755 and EU414756 (ITS1, 5.8S, ITS2), and KP205570 (LSU, D1/D2 regions). Since intragenomic variation in ITS1 sequence is present, the ITS1 sequence *B. eastonii* is defined as the least inclusive clade containing both EU414755 and EU414756. The type culture (isolate GE09) is stored cryogenically in liquid nitrogen at Aberystwyth University. Type material from 3 d old cultures and preserved in 5% glutaraldehyde is lodged in the biorepositories at: Aberystwyth University (code ABS) with isotype material at Royal Botanic Gardens, Kew, London (K); and Friedrich-Schiller-Universität Jena, Germany (JE).

Etymology. From the Welsh words for large cow ('buwch fawr'), since the original isolate GE09 was isolated from a buffalo for which there is no Welsh word. The specific epithet in honour of our former colleague Gary Easton who isolated this fungus.

Nomenclature. *Buwchfawromyces* Callaghan, Tony & G.W. Griff., gen. nov. Strictly anaerobic fungus forming a monocentric thallus with a single sporangium, usually borne on a swollen sporangiophore connected to a branching and twisted rhizoidal system. Zoospores are spherical and uniflagellate. "*Buwch fawr*" means large cow in Welsh.

Conclusions

It has been apparent since the widespread use of DNA sequence data to identify anaerobic fungi that many of the sequences currently lodged with GenBank do not fall into any of the currently recognised genera of the Neocallimastigomycota. The situation is further complicated by the presence of many sequences from isolates which are very likely misidentified, a phenomenon which is also problematic for other groups of Fungi (Schoch et al. 2014).

A new genus is described based on on a pure culture which was isolated a decade ago and for which ITS1 sequence has been lodged with GenBank since 2008. The RefSeq project aims to resolve this, and other related issues, by linking ITS and LSU sequences from vouchered reference specimens to accepted names (Schoch et al. 2014). As shown above, ITS sequences for anaerobic fungi can be problematic due to intragenomic sequence variation. Similar problems have not been found for the more conserved LSU region of the rRNA locus (Dagar et al. 2011; Eckart et al. 2010), highlighting the synergy of using both loci. We also note that analysis of LSU data does have the distinct advantage of yielding robust alignments across a wide range of basal fungal taxa (including Chytridiomycetes) and being amenable to direct sequencing of PCR products.

Acknowledgements

TMC is grateful to the Aberystwyth University Postgraduate Research Studentship Scheme for funding. JEE gratefully acknowledges funding from BBSRC (grant code BBS/E/W/10964A01). SSD and AKP are grateful respectively for the awarding of a Stapledon Memorial Trust Travelling Fellowship and DBT-CREST Award Fellowships, which permitted research visits to IBERS, Aberystwyth University. SP and DH are grateful to Universität Innsbruck and Technische Universität Berlin for the award of travel scholarships. We are also grateful to Dr. Paul Kirk, RBG Kew for nomenclatural advice.

References

Breton A, Bernalier A, Dusser M, Fonty G, Gaillard-Martinie B, Guillot J (1990) *Anaeromyces mucronatus* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus. FEMS Microbiology Letters 58: 177–182.

Brookman JL, Ozkose E, Rogers S, Trinci APJ, Theodorou MK (2000) Identification of spores in the polycentric anaerobic gut fungi which enhance their ability to survive. FEMS microbiology ecology 31: 261–267. doi: 10.1111/j.1574-6941.2000.tb00692.x

Chen Y-C, Hseu R-S, Chein C-Y (2002) *Piromyces polycephalus* (Neocallimastigaceae), a new rumen fungus. Nova Hedwigia 75: 409–414. doi: 10.1127/0029-5035/2002/0075-0409

Dagar SS, Kumar S, Mudgil P, Singh R, Puniya AK (2011) D1/D2 domain of large-subunit ribosomal DNA for differentiation of *Orpinomyces* spp. Applied and environmental microbiology 77: 6722–6725. doi: 10.1128/AEM.05441-11

Davies DR, Theodorou MK, Lawrence MIG, Trinci APJ (1993) Distribution of anaerobic fungi in the digestive tract of cattle and their survival in feces. Journal of General Microbiology 139: 1395–1400. doi: 10.1099/00221287-139-6-1395

Day JP, Kell DB, Griffith GW (2002) Differentiation of *Phytophthora infestans* sporangia from other airborne biological particles by flow cytometry. Applied and environmental microbiology 68: 37–45. doi: 10.1128/AEM.68.1.37-45.2002

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11–15.

Drummond A, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, A W (2011) Geneious v5.4. http://www.geneious.com/

Eckart M, Fliegerová K, Hoffmann K, Voigt K (2010) Molecular Identification of Anaerobic Rumen Fungi. In: Gherbawy Y, Voigt K (Eds) Molecular Identification of Fungi. Springer Berlin Heidelberg, 297–313. doi: 10.1007/978-3-642-05042-8_14

Edwards J, Huws S, Kingston-Smith A, Jimenez H, Skøt K, Griffith GW, McEwan NR, Theodorou MK (2008) Dynamics of initial colonisation of non-conserved perennial ryegrass by anaerobic fungi in the bovine rumen. FEMS microbiology ecology 66: 537–546. doi: 10.1111/j.1574-6941.2008.00563.x

Fliegerová K, Mrazek J, Voigt K (2006) Differentiation of anaerobic polycentric fungi by rDNA PCR-RFLP. Folia Microbiologica 51: 273–277. doi: 10.1007/BF02931811

Fliegerová K, Voigt K, Kirk PM (2012) *Caecomyces hurleyensis* (Theodorou & J. Webb) Fliegerová, K. Voigt & P.M. Kirk, comb. nov. IF550013. Index Fungorum 1: 1.

Frey W (2012) Syllabus of plant families. Part 1/1: blue-green algae, myxomycetes and myxomycete-like organisms, phytoparasitic protists, heterotrophic heterokontobionta and fungi. Ed. 13. Gebrüder Borntraeger Verlagsbuchhandlung.

Griffith GW, Baker S, Fliegerova K, Liggenstoffer A, van der Giezen M, Voigt K, Beakes G (2010) Anaerobic fungi: Neocallimastigomycota. IMA Fungus 1: 181–185. doi: 10.5598/imafungus.2010.01.02.11

Griffith GW, Shaw DS (1998) Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. Applied and environmental microbiology 64: 4007–4014.

Gruninger RJ, Puniya AK, Callaghan TM, Edwards JE, Youssef N, Dagar SS, Fliegerova K, Griffith GW, Forster R, Tsang A (2014) Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. FEMS microbiology ecology. doi: 10.1111/1574-6941.12383

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk WOG (2010) New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biology 59: 307–321. doi: 10.1093/sysbio/syq010

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson O-E, Huhndorf S, James TY, Kirk PM, Lucking R, Lumbsch T, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde K, Ironside JE, Koljalg U, Kurtzman CP, Larsson K-H, Lichwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schussler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N (2007) A higher-level phylogenetic classification of the fungi. Mycological Research 111: 509–547. doi: 10.1016/j.mycres.2007.03.004

Ho YW, Barr DJS, Abdullah N, Jalaludin S (1993a) *Anaeromyces*, an earlier name for *Ruminomyces*. Mycotaxon 47: 283–284.

Ho YW, Barr DJS, Abdullah N, Jalaludin S, Kudo H (1993b) *Piromyces spiralis*, a new species of anaerobic fungus from the rumen of goat. Mycotaxon 48: 59–68.

Ho YW, Bauchop T, Abdullah N, Jalaludin S (1990) *Ruminomyces elegans* gen. et sp.nov., a polycentric anaerobic rumen fungus from cattle. Mycotaxon 38: 397–405.

James TY, Letcher PM, Longcore JE, Mozley-Standridge SE, Porter D, Powell MJ, Griffith GW, Vilgalys R (2006) Chytridiomycota: 14 clades of flagellated Fungi. Mycologia 98: 860–871. doi: 10.3852/mycologia.98.6.860

Joblin KN (1981) Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. Applied and environmental microbiology 42: 1119–1122.

Katoh K, Misawa K, Kuma KÄ, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic acids research 30: 3059–3066. doi: 10.1093/nar/gkf436

Kirk PM (2012) *Anaeromyces polycephalus* (Chen YC, Chien CY, Hseu RS) Flieg., K. Voigt & P.M. Kirk. IF550012. Index Fungorum 1: 1.

Kittelmann S, Naylor GE, Koolaard JP, Janssen PH (2012) A proposed taxonomy of anaerobic fungi (class Neocallimastigomycetes) suitable for large-scale sequence-based community structure analysis. PLoS ONE 7: e36866. doi: 10.1371/journal.pone.0036866

Koetschan C, Kittelmann S, Lu J, Al-Halbouni D, Jarvis GN, Müller T, Wolf T, Janssen PH (2014) Internal Transcribed Spacer 1 Secondary Structure Analysis Reveals a Common Core throughout the Anaerobic Fungi (Neocallimastigomycota). PLoS ONE 9: e91928. doi: 10.1371/journal.pone.0091928

Koljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Duenas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Luecking R, Martin MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Poldmaa K, Saag L, Saar I, Schuessler A, Scott JA, Senes C, Smith ME, Suija A, Taylor DL, Telleria MT, Weiss M, Larsson KH (2013) Towards a unified

paradigm for sequence-based identification of fungi. Molecular Ecology 22: 5271–5277. doi: 10.1111/mec.12481

Li JL, Heath IB, Bauchop T (1990) *Piromyces mae* and *Piromyces dumbonica*, 2 new species of uniflagellate anaerobic chytridiomycete fungi from the hindgut of the horse and elephant. Canadian Journal of Botany 68: 1021–1033. doi: 10.1139/b90-129

Liggenstoffer AS, Youssef NH, Couger MB, Elshahed MS (2010) Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME Journal 4: 1225–1235. http://www.nature.com/ismej/journal/v4/n10/suppinfo/ismej201049s1.html

McGranaghan P, Davies JC, Griffith GW, Davies DR, Theodorou MK (1999) The survival of anaerobic fungi in cattle faeces. FEMS microbiology ecology 29: 293–300. doi: 10.1111/j.1574-6941.1999.tb00620.x

Milne I, Wright F, Rowe G, Marshall DF, Husmeier D, McGuire G (2004) TOPALi: software for automatic identification of recombinant sequences within DNA multiple alignments. Bioinformatics 20: 1806–1807. doi: 10.1093/bioinformatics/bth155

Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson K-H (2008) Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evolution Bioinformatics Online 4: 193–201.

Nilsson RH, Ryberg M, Sjokvist E, Abarenkov K (2011) Rethinking taxon sampling in the light of environmental sequencing. Cladistics 27: 197–203. doi: 10.1111/j.1096-0031.2010.00336.x

Orpin CG (1975) Studies on the rumen flagellate *Neocallimastix frontalis*. Journal of General Microbiology 91: 249–262. doi: 10.1099/00221287-91-2-249

Orpin CG (1977) Rumen flagellate *Piromonas communis* - Its life history and invasion of plant material in rumen. Journal of General Microbiology 99: 107–117. doi: 10.1099/00221287-99-1-107

Ozkose E (2001) Morphology and molecular ecology of anaerobic fungi. Ph.D.: University of Wales, Aberyswyth.

Pawlowska TE, Taylor JW (2004) Organisation of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature, London 427: 733–737. doi: 10.1038/nature02290

Powell MJ, Letcher PM (2014) Chytridiomycota, Monoblepharidomycota and Neocallimastigomycota. In: Esser K (Ed.) The Mycota VII Systematics and Evolution PartA. Springer, 141–175.

Pringle A, Moncalvo J-M, Vilgalys R (2000) High levels of variation in ribosomal DNA sequences within and among spores of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. Mycologia 92: 259–268. doi: 10.2307/3761559

Sakurada M, Tsuzuki Y, Morgavi DP, Tomita Y, Onodera R (1995) Simple method for cryopreservation of an anaerobic rumen fungus using ethylene-glycol and rumen fluid. FEMS Microbiology Letters 127: 171–174. doi: 10.1111/j.1574-6968.1995.tb07469.x

Schoch CL, Robbertse B, Robert V, Vu D, Cardinali G, Irinyi L, Meyer W, Nilsson RH, Hughes K, Miller AN (2014) Finding needles in haystacks: linking scientific names, reference specimens and molecular data for Fungi. Database bau061. doi: 10.1093/database/bau061

Struchtemeyer CG, Ranganathan A, Couger MB, Liggenstoffer AS, Youssef NH, Elshahed MS (2014) Survival of the anaerobic fungus *Orpinomyces* sp. strain C1A after prolonged air exposure. Scientific Reports 4: doi: 10.1038/srep06892

Yamamoto N, Bibby K (2014) Clustering of fungal community internal transcribed spacer (ITS) sequence data obscures taxonomic diversity. Environmental Microbiology. doi: 10.1111/1462-2920.12390

Yarlett NC, Yarlett N, Orpin CG, Lloyd D (1986) Cryopreservation of the anaerobic rumen fungus *Neocallimastix patriciarum*. Letters In Applied Microbiology 3: 1–3. doi: 10.1111/j.1472-765X.1986.tb01533.x

Supplementary material 1

SuppFig. 1

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Adobe PDF file

Explanation note: Putative fused zoospores in isolate GE09 (A) and the similar structure reported by Orpin (1975) (B). Swollen sporangiophores and twisted rhizoids, as found in isolate GE09, were also reported for *Piromyces spiralis* (C, D) by Ho et al. (1993), whilst *Anaeromyces* (formerly *Piromyces*) *polycephalus* also forms a swollen sporangiophore (E). In older cultures of GE09, thick walled putative spore structures were frequently observed (F, G). Scalebar indicates 20 μm. A video of the putative fused zoospore is found at: https://www.youtube.com/watch?v=im14hz1jiX0.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Supplementary material 2

SuppFig. 2

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Adobe PDF file

Explanation note: Alignment of part of the ITS1 region across a range of anaerobic fungi from all the known genera. The sequences of the modified MN100 primer used by Liggenstoffer et al. (2010) (TCCTACCCTTTGTGAATTTG) is indicated (green). For all clades except *Buwchfawromyces*, there is a good match for this primer. However, for members of the *Buwchfawromyces*, there are several mismatches at the 3' end of the primer binding site which are likely to impede PCR amplification.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Supplementary material 3

SuppFig. 3

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Adobe PDF file

Explanation note: Intragenomic variation in ITS sequences among the five clones sequenced from isolate GE09. 18S boundary ends with GATCATTA and 5.8S begins with CAACTTT, according to the convention of Hibbett et al. (1995).

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Supplementary material 4

SuppTable 1

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Microsoft Excel file

Explanation note: Details of ITS sequences falling into the *Buwchfawromyces* clade. The first five are all derived from the isolate GE09.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.